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Evaluation of antibody against hydatid fluid, protoscolex and adult worms of *Echinococcus granulosus* antigens by ELISA in mice

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Abstract

Echinococcosis is one of the world's relevant zoonoses, affecting both humans and their domestic animals and caused by infection with the metacestode stage of the dog tapeworm, *Echinococcus granulosus*. In the present study, twenty mice were randomly divided into 4 groups of 5. Each mouse in groups 1, 2 and 3 was immunized with 100 μg of hydatid cyst fluid protein, protoscolex and adult worms of *E. granulosus* proteins in 100 μl of phosphate-buffered saline which was emulsified with an equal volume of Freund's complete adjuvant respectively. In group 4, mice were just immunized with above adjuvant in PBS. Mice were boosted four weeks after the first immunization with the same preparation and with Freund's incomplete adjuvant. Three weeks later, each mouse was challenged with 2000 protoscolices intraperitoneally. Sera were collected before and after each immunization and serum antibodies were tested by ELISA. Results showed that the level of antibody in mice which were immunized with adult worms of *E. granulosus* on day 28 was roughly seven times higher than before immunization and was higher than hydatid cyst fluid and protoscolex. Level of antibody in mice immunized with adult worms of *E. granulosus* was also higher than in hydatid cyst fluid and protoscolex groups at day 49. The results of this article indicate that antigen of adult worms of *E. granulosus* can be used as a candidate for production of a vaccine.

Key words: *Echinococcus granulosus,* antibody, ELISA, mouse.

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Introduction

Echinococcosis is one of the most widespread parasitic zoonoses in the world geographically, with transmission occurring in tropical, temperate and arctic biomes. Most human infestations are due to *Echinococcus granulosus* (*E. granulosus*) transmitted between domestic dogs and livestock. But this cosmopolitan species also cycles between wild carnivores (principally canids) and wild ungulates (Craig et al., 2003). Hydatid cysts of *E. granulosus* develop in internal organs (mainly the liver and lungs) of humans and intermediate hosts such as sheep, horses, cattle, pigs, goats and camels (Lahmar et al., 2004) as unilocular fluid filled bladders (Zhang et al., 2003 a). The disease is a major public health problem in sheep-raising regions of the World (Moro et al. 2005). Taeniid cestode antigens (derived from adult worms, juvenile intestinal stages or oncospheres) interact with the immune system of the host and may lead to the production of specific antibodies. It is generally accepted that *Echinoccocus* spp is unaffected by the immune response during the developing stage (Eckert and Deplazes, 2005). Enzyme-linked immunosorbent assay (ELISA) based on detection of these antibodies has been developed for diagnosis of *Echinococcus* infection in definitive hosts (Jenkins and Rickard, 1986). Over the past 30 years the use of human infections has been very evident, largely through the detection of parasite-specific antibody in the serum. In the last decade more advanced approaches such as circulating antigen detection, lymphocyte proliferation responses and cytokine analyses and molecular techniques (Siles-Lucas and Gottstein, 2001) have been applied to provide useful information on these infections. However, it is still antibody detection which is the most widely used for confirmation of clinical diagnosis and in epidemiological surveys (Craig *et al*., 2003). Furthermore, experimental infections of mice with eggs or oncospheres of *E. granulosus* showed that susceptibility varies with different strains of mice. *In vitro* experiments have also shown that neutrophils, in association with antibodies, can bring about the killing of *E. granulosus* oncospheres, suggesting a possible role for antibody-dependent cell-mediated cytotoxicity reactions. At the early stages of disease, there is a marked activation of cell mediated immunity to the parasite (Fotiadis *et al.,* 1999). The propose of this study was to determine whether immunization of mice with three antigens might induce the humoral immune response and antibody production against hydatid fluid, protoscolices and adult *E. granulosus* worm antigens.

Materials and Methods

Samples preparation

Sheep hydatid cysts were collected from Mashhad slaughterhouse. Hydatid cyst fluid (HCF) was aspirated from cysts in the liver and lungs and centrifuged at 5000 g for 30 min at 4° C to remove protoscolices (Pr) and stored at -20°C until used. Protoscolices were washed 3-times with hank's solution. Sample was freeze-thawed 3 times and mixed with four volumes of PBS, pH 7.4, containing sodium azide at 0.1 mg/ml. Sample was then sonicated at 110 V, 170 W ultrasonic disintegrator (Hielscher, Germany), for 3x15 seconds on ice. The preparation was then left on ice for one hour and centrifuged for 30 min at 10,000 g and then filtered (0.22 µm) (Ahmad et al. 2001). Soluble protein of adult worms of *E. granulosus* was also prepared (Hashemi Tabar et al. 2005). Briefly, two hundred of mature *E. granulosus* which were kept in 10% formaline for 8 months at room temperature (RT) to insure that the adult worms were killed, and then were washed three times with Hanks solution. After washing with phosphate buffer saline (PBS) [pH = 7.3], freeze-thawed three times in liquid nitrogen and 42ºC, sample was then homogenized in a blender and sonicated as described above. Finally, the sample was filtrated with 0.22 µm filter. Proteins concentration were measured by Bradford (1976) method and kept at -20 º C until used.

Immunization and Challenge

Twenty mice were randomly divided into 4 groups of 5 mice (three immunized and one control group). The mice in groups 1, 2 and 3 received 100 µg (100 µl) of HCF, Pr and adult worms of *E. granulosus* antigens plus 100 µl of Freund's complete adjuvant (FCA), respectively. Mice in control group were immunized with adjuvant in PBS. Second immunization was conducted after four weeks with the same preparation except that FCA was replaced by Freund's incomplete adjuvant (FIA). Three weeks after the second immunization, each mouse was challenged with 2000 protoscolices intraperitoneally as described previously (Hashemi Tabar *et al*., 2005; 2006). Mice were sacrificed by CO_2 seven months post challenge. Blood samples were collected before and after each immunization from mice and sera were separated by centrifugation at x12000 g for 3 min after placing the samples at 4°C overnight. Sera were stored at -20°C until used.

ELISA

To screen the activity of antibody against three types of protein antigens (hydatid fluid, protoscolices and adult *E. granulosus* worm antigens), ELISA was carried out as described by Wen and Craig (1994). Assays were used in 96-well micro-titer plates (Maxisorp). All the solutions were used at 100 µl per well, except the blocking solution which was used at 300 µl. The wells were washed four times with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.2) containing 0.1% v/v of Tween-20. Appropriate wells were coated with 5 µg of HF, Pr and adult worms of *E. granulosus* proteins in 100 µl of 0.1 M $NaHCO_3$ and the plates were then left exposed to air overnight at RT to allow the solution to evaporate into dryness. The following day, after washing, wells were blocked with 300 µl of 2% (w/v) bovine serum albumin (BSA) in PBS and then incubated for 2 h at RT to block any remaining unblocked attachment sites on the wells. After washing the wells, diluted sera of mice were added and then the plates incubated 1 h at RT. The plate was washed and then the secondary antibody, sheep anti-mouse IgG phosphatase (Sigma, USA) was added at 1:2000 dilutions into all the wells and incubated for 1 h at RT. The plate was washed as described above to remove the excess conjugate. For color development, 100 µl of TMB was added to each well as a substrate and the reaction was terminated after 15 min by the addition of 100 µl of 1M of HCL solution to each well. The absorbance at 490 nm was monitored in ELISA reader (Bio-Tek Instruments).

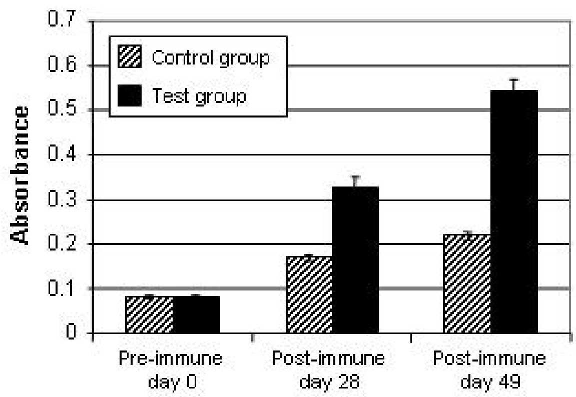


Figure 1: Mean absorbance of test and control groups on day 0, 28 and 49. Day 0, 28 and 49 indicate per-immune, four weeks after first immunization and three weeks after second immunization respectively. Mice in test group were immunized with hydatid cyst fluid. Values are listed as: Control group: day 0, Mean ± SEM= 0.08±0.0042; day 28, Mean ± SEM= 0.017±0.008; day 49, Mean ± SEM= 0.22±0.0092; and Test group: day 0, Mean ± SEM= 0.08±0.0037; day 28, Mean ± SEM= 0.33±0.0218; day 49, Mean ± SEM= 0.54±0.0301.

Statistical analysis

The results of these experiments were analyzed by Non parametric t-test and Repeat measure using SPSS software version 9. Significant association were identified when P-value of less than 0.05 were observed (Daly et al., 1992).

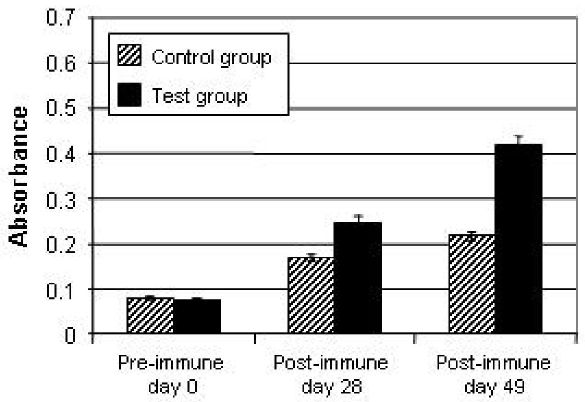


Figure 2: Mean absorbance of test and control groups on day 0, 28 and 49. Day 0, 28 and 49 indicate per-immune, four weeks after first immunization and three weeks after second immunization respectively. Mice in test group were immunized with protoscolices. Values are listed as: Control group: day 0, Mean ± SEM= 0.08±0.0042; day 28, Mean ± SEM= 0.017±0.008; day 49, Mean ± SEM= 0.22±0.0092; and Test group: day 0, Mean ± SEM= 0.08±0.0036; day 28, Mean ± SEM= 0.25±0.0173; day 49, Mean ± SEM= 0.42±0.0169.

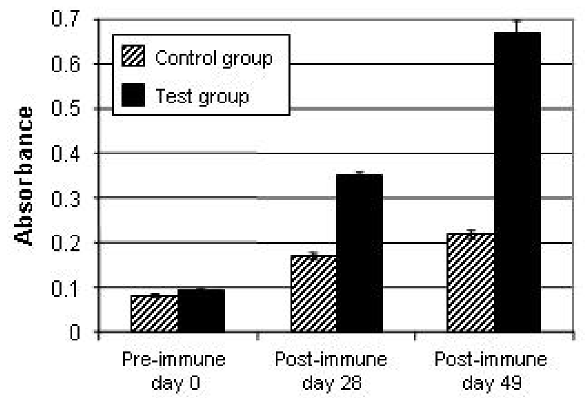


Figure 3: Mean absorbance of test and control on day 0, 28 and 49. Day 0, 28 and 49 indicate per-immune, four weeks after first immunization and three weeks after second immunization respectively. Mice in test group were immunized with whole body of *E. granulosus.* Values are listed as: Control group: day 0, Mean ± SEM= 0.08±0.0042; day 28, Mean ± SEM= 0.017±0.008; day 49, Mean ± SEM= 0.22±0.0092 and Test group: day 0, Mean ± SEM= 0.09±0.0041; day 28, Mean ± SEM= 0.35±0.0076; day 49 Mean ± SEM= 0.67±0.0271.

Results Results showed that the level of antibody production was different by use of three different antigens and in all of these groups (mice which were immunized with hydatid cyst fluid, protoscolex and adult worms of *E. granulosus* proteins), level of antibody was significantly higher (P< 0.05) than control group (Fig.1, a. b. and c.). The mice immunized with HCF produced higher antibody (P< 0.05) than mice immunized with Pr on day 28 (four weeks after the first immunization). Whereas antibody produced by adult worms of *E. granulosus* on day 28 was roughly seven times more than before immunization (P< 0.05). Level of antibody on day 49 (3 weeks after the second immunization) in mice immunized with HCF was higher than in Pr group (P< 0.05). Also, the level of antibody in mice immunized with adult worms of *E. granulosus* was higher than in HCF and Pr groups on day 49, and these results showed similar trends to the results of protection with three antigens (P< 0.05) (Fig.2).

Discussion

In our study, the levels of antibody production were evaluated in mice against three antigens. Mice were immunized with Pr, did not produce high level of antibody four weeks after the first immunization, whereas antibody produced by HCF and adult worms of *E. granulosus* was roughly 5 and 7 times of before immunization, respectively. Lin et al., (2004) reported that the specific IgG was induced during the 3rd week and continued to increase until week 10. Although the level of antibody on day 49 was very high in all three groups, but the level of antibody in mice immunized with adult worms of *E. granulosus* was much higher than in HCF and Pr groups. It is suggested that this antigen is a good candidate for protection against hydatid cyst. Infection of BALB/c mouse with protoscolices of *E. granulosus* constitutes a model for the study of secondary hydatidosis and the associated immune response in immunization and infection trials (Khaled et al., 2005). Immunogenicity of two *Echinococcus granulosus* antigens EgA31 and EgTrp in mice were investigated by ELISA and these antigens elicited high antibody titer and both antigens separately or in combination as candidate vaccine proteins is discussed (Fraize

et al., 2005). It has been shown by (Shi et al., 2009) that rEgP-29 induced high levels of specific antibodies in mice after the third immunization. The protective efficacy of humoral immunity in *E. granulosus* was not only correlated with the level of IgG, but also associated with the isotype of IgG.

It has been reported that antibody response was followed during 68 weeks in 17 BALB/c mice intraperitoneally infected with *E. granulosus* protoscolices (PSC) and in three mice immunized with dead PSC (Severi et al., 1997). Infected mice showed similar profiles of specific IgG and IgM with maximum titers from week 38 to 53. Immunized mice did not show significant levels of specific IgM and after week 15, showed IgG titers to be lower than the infected mice. It has been reported that mice immunized against *E. granulosus* secondary infection with protoscolices which were subjected to ultraviolet irradiation but were not killed and these mice were evaluated as having high level of antibody (Molan and Saeed, 1988). Zhang et al., (2003b) showed that mice produced lower levels of antibodies than of a secondary challenge infection given three weeks later by a different route (intraperitoneal, subcutaneous or intravenous injection). Most mice did not evoke significant antibody responses against oncospheral antigens until five weeks post-infection (PI). Level of IgG, especially IgG1 against oncospheral antigens increased from week four PI, to a maximum at week 9 PI. In addition, antibodies against HCF antigens increased at the same time as the recognition of oncospheral antigens. A higher level of immunity was achieved in sheep against *E. granulosus* using oncosphere antigen (Heath and Lawrence, 1996) Antigens derived from cyst fluid (Heath *et al*., 1992) and protoscolices (Hernandez and Nieto, 1994) have been used against *E. granulosus* and mice produced a higher level of antibody against both antigens. Although three antigens have been described in this paper can be used for ELISA test, but it is necessary to study and compare the specificity and sensitivity of these antigens in diagnosis of hydatid cyst.

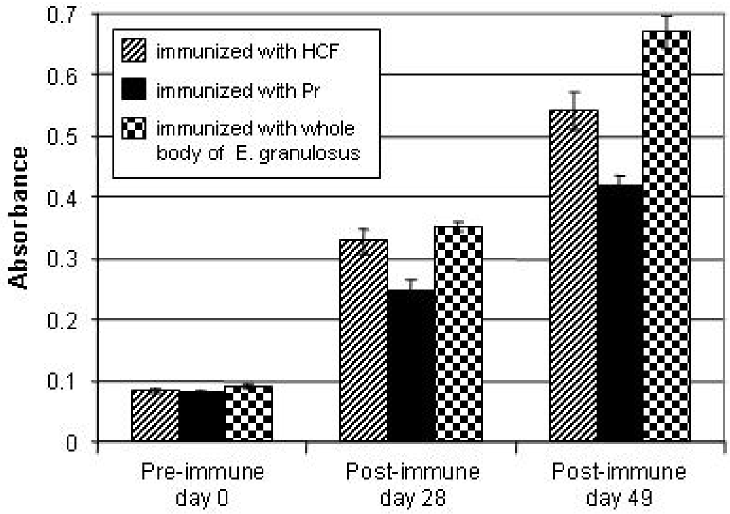


Figure 4: Mean absorbance of 3 test groups on day 0, 28 and 49. Day 0, 28 and 49 indicate per-immune, four weeks after first immunization and three weeks after second immunization respectively.

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ارزیابی تولیدآنتی بادی علیه آنتی ژنهای مایع هیداتید؛ پروتواسکولکس و انگل بالغ *اکینوکوکوس گرانولوزوس* با استفاده از روش الایزا درموش

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چکیده

هیداتیدوز یکی ازبیماریهای مشترک بین انسان و دام با انتشار جهانی است و بوسیله مرحلـه لاروی انگـل اکینوکوکـوس گرانولـوزوس ایجاد مشود.

در مطالعه حاضر، ۲۰ موش به چهار گروه ۵ تایی تقسیم شدند (سه گروه آزمایش و یک گروه کنترل). هر موش در گروههای آزمایـشی ۱ ، ۲ و ۳ بوسیله ۱۰۰ میکروگرم (۱۰۰ میکرو لیتر) آنتی ژنهای مایع کیست هیداتید، پروتواسکولکس و انگل بالغ *اکینوکوکوس گرانولوزوس* مورد تزریق قرار گرفتند. به هر موش ۲۰۰ میکرولیتر (۱۰۰ میکروگرم از آنتی ژنهای فوق در ۱۰۰ میلی لیتر فسفات بافر حل شده و با ۱۰۰ میکرو لیتر ادجوانت کامل فروند مخلوط شدند) به روش زیر جلدی تزریق گردید. موشهای گروه کنترل نیز با دوز یکسان (۱۰۰ میکرو لیتـر فسفات بافر و ۱۰۰ میکرو لیتر ادجوانت فوق) مورد تزریق قرار گرفتند. تزریق مجدد ۴ هفته بعد با مقدار فوق و با استفاده از ادجوانت نـاقص فروند انجام شد. سه هفته بعد، هر موش با ۲۰۰۰ عدد پروتواسکولکس به روش داخل صفاقی تلقیح شدند. خونگیری از موشها قبل و پس از تزریقات انجام شد و میزان آنتی بادی بوسیله آزمایش الایزا مورد بررسی قرار گرفت.

سطح تولید آنتی بادی در ۲۸ روز پس از تزریق در موشهایی که با انگل بالغ ایمن شده بودند ۷ برابر بیشتر قبل از ایمن کردن آنها بود و نیز تولید آنتی بادی در این گروه بیشتر از موشهای ایمن شده با مایع کیست هیداتید و پروتواسکولکس بود. سطح تولیـد آنتـی بـادی در روز ۴۹ (سه هفته پس از دومین ایمن سازی) در گروه آزمایشی که با آنتی ژن انگل بالغ *اکینوکوکوس گرانولوزوس* مورد تزریق قرار گرفته بودند بیشتر از دو گروه دیگر بود. نتایج حاصله از این آزمایش دلالت بر این دارد که آنتی ژن انگل بـالغ *اکینوکوکـوس گرانولـوزوس* میتوانـد بـه عنوان یک کاندیدا برای تولید واکسن مورد استفاده قرار گیرد.

کلمات کلیدی : *اکینوکوکوس گرانولوزوس*، آنتی بادی، الایزا، موش